CYTOLOGICAL STUDIES DURING GERMINAL VESICLE BREAKDOWN OF PECTINARIA GOULDII WITH VITAL DYES, CENTRIFUGATION AND FLUORESCENCE MICROSCOPY [1]

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It is becoming increasingly evident that subcellular cytoplasmic particles probably play an important part in the embryonic differentiation of the egg. The possible role of these entities in differentiation of the marine egg is aptly reviewed by Brachet (1957), Gustafson (1954), Raven (1958, 1961) and Shaver (1957).

One approach has been to identify and then trace the assignment of certain particulate groups during the early recognizable states in morphogenesis.

In the living egg innumerable investigations have used centrifugal forces to localize and subsequently identify the cell particulates by stratification. The early experiments by Lillie (1906, 1909) on the annelids *Chaetopterus* and *Nereis*, and of Morgan (1908, 1909, 1910) on *Arbacia* indicated that centrifugal force would stratify granules of yolk, pigment, mitochondria and other cytoplasmic particles according to their specific gravity.

In his classic paper on the egg of *Chaetopterus* Lillie (1906) observed the subcellular particulates in the living and fixed egg. With centrifugation of 1500 to 2000 rpm. he found that the endoplasm was stratified into a small grey cap, a clear band and a yellow hemisphere.

During germinal vesicle breakdown he noted the release of a residual substance composed of spherules and microsomes which he believed formed the grey centripetal cap. He also noted that the microsomes changed from an acid to a basic state as they moved into the cytoplasm and he followed their distribution during early cleavage.

More recent studies, using sucrose layering and much higher centrifugal forces, by Harvey (1939) on *Chaetopterus* and Costello on *Nereis* (1939, 1958) have contributed greatly toward identification of the various layers stratified in the annelid egg. Stratification in the egg of *Arbacia* (Harvey, 1941) was very similar to the annelid egg; in *Sphaerechinus granularis* the various strata obtained were almost identical to those obtained in the egg of *Chaetopterus* (Harvey, 1939). Thus a close comparison between the stratification of these eggs and the closely related eggs of *Pectinaria* seemed warranted.

Other observers have used intravital dyes in combination with centrifugation to identify cell particles in living eggs (Lucké, 1925; Harvey, 1941; Iida, 1942; Monné, 1944; Tweedell, 1960b).

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Further investigations have demonstrated a close relationship between certain vitally stained particles, such as the mitochondria, and cleavage activity of the egg. This linkage has been established in eggs of the sea urchin by Iida (1942) and Kojima (1959a), in *Urechis unicinctus* (Kojima, 1959b) and in the mollusc, *Spisula solidissima* by Rebhun (1959, 1960).

One role of mitochondria in differentiation of the ascidian eggs, *Beroë ovata* and *Phallusia mamillata,* has been verified. Here segregation of the mitochondria during development has been traced with Janus green. Subsequently, the mitochondria were found to be involved in the formation of the ciliary plates of the larvae. Significantly, the localized activity of the mitochondrial enzymes, cytochrome oxidase and succinic dehydrogenase, has been correlated with the Janus green staining of the mitochondria (Reverberi, 1956, 1957a, 1957b).

The pale yellow eggs of *Pectinaria gouldii* are extremely useful for cytological studies of this type because of their unusual transparency and relatively small amount of yolk. As a result, cytoplasmic and nuclear particles remain visible during development of the living egg.

In order to recognize and chemically identify the many particulates in the egg protoplasm of *Pectinaria*, numerous vital dyes and vital fluorochromes were applied to the living egg both before and after germinal vesicle breakdown. In addition to observations on these eggs, other eggs were centrifuged to separate and identify particles by their stratified position and staining characteristics. Further cytochemical tests on whole fixed eggs helped verify these findings.

Materials and Methods

The marine polychaete annelid, *Pectinaria* (*Cistenides*) *gouldii* Verrill, after Hartman (1941), is found in the mud-sand flats beneath shallow water beyond the low tide mark. This fascinating worm lives in a beautiful cone-shaped tube constructed of fitted sand grains, one grain in thickness, that are cemented together with secretions from the cementing organ (Watson, 1928).

Specimens for this study were obtained from the Woods Hole area by the Marine Biological Laboratory Supply Department. In the laboratory they were kept either in a large container of rapidly running sea water or in a shallow tray of sand that was submerged under running sea water. The animals usually burrowed with the head downward, as in nature, and the tapered end of the tube extended above the surface, especially at night. The adults did not survive long in the laboratory once they were removed from their tubes.

Prior to shedding, the eggs of *Pectinaria* are freely suspended within the coelomic fluid where they constantly shift back and forth with the pistonlike movement of the adult female. Many developmental stages of the oocyte are also present in the fluid. Following mechanical stimulation of the female within the tube, the coelomic fluid and eggs are vigorously shed into the sea water where the mature eggs continue maturation with breakdown of the germinal vesicle (GV). In the male, sperm packets are released in an identical fashion.

Eggs so obtained were studied immediately after shedding, with the bright field and dark field microscope. Other egg aliquots were shed directly into vital dyes prepared in sea water to stain various particulates. Alternatively, eggs were dyed with vital fluorochromes and observed under the fluorescence microscope.

Wherever possible, the specific vital dyes used were designated according to the revised color index numbers (CI).

Application of a single vital dye to the living egg often resulted in staining of more than one constituent of the protoplasm. For this reason both the untreated and dyed eggs were centrifuged in order to stratify and differentiate between identically dyed particles. Thus particles could be classified from their relative stratum after centrifugation and from numerous cross-checks with different dyes or combinations of microscopy.

Because of the preparatory time, the mature eggs had already undergone GV breakdown at the end of centrifugation but immature eggs were useful for studying particle distribution in pre-breakdown stages. Immature eggs did not undergo germinal vesicle breakdown on contact with sea water.

The eggs were layered over fresh sucrose (0.75 molar) in plastic centrifuge tubes, in the proportion of one-fifth sucrose to four-fifths eggs and sea water. The tubes were then placed in an ice bath prior to centrifugation. The eggs were centrifuged either in a refrigerated centrifuge (0° C.) at 3,000 to 4,850 *G* for one hour, in a high speed centrifuge at 17,000 *G* for 30 minutes at 4° C., or in an insulated preparatory ultracentrifuge (6° C.) at 33,000 *G* for 12 minutes. This enabled much sharper separation of granular bands than centrifugation at room temperatures.

After centrifugation, the eggs were immediately placed in an ice bath until the time of observation since lack of refrigeration allowed rapid "return" of the stratified components.

For all cytological observations the eggs were transferred in a small drop to the polished depression of a thin micro culture slide. A coverslip was then placed over the well so that the drop was in contact with the depression and the coverslip.

The light source for both standard dark field and fluorescence microscopy of the living eggs was a mercury vapor lamp (G. E. H4AB). The Osram HBO 200W bulb was found too intense for extended observations of living eggs.

In dark field observations a noviol "O" filter (Corning no. 3060) was used as an exciter filter at the light source to remove most ultraviolet, and a distilled water cell or an infrared cut-off filter (Kodak no. 301) for absorbing the infrared.

For blue light fluorescence (dark field) the infrared cut-off filter and a violet exciter filter (Corning no. 5113) were coupled to one of two barrier filters, a yellow shade (Corning no. 3486) filter which permits observation of fluorescence above 5100 Å or a noviol "A" filter (Corning no. 3389) that transmits down to 4200 Å.

Ultraviolet-induced fluorescence (dark field) was produced with either a blue purple ultra filter (Corning no. 5850), a red purple corex A (Corning no. 9863) or a Wratten 18A filter (Kodak). The same barrier and heat filters were used. Selection of filters for fluorescence depended upon the properties of the particular fluorochrome used.

A standard binocular microscope was equipped with a front surfaced aluminized mirror. Special funnel stops were used in the 20 ×, 40 × and 97 × objectives for all standard dark field and fluorescence observations, to reduce haloing from the relatively thick eggs.

Certain cytochemical reactions cannot be shown with vital dyes. In order to broaden the chemical tests and substantiate those obtained from the eggs stained

in vitro, eggs were fixed at 30-second intervals from the time of shedding to 20 minutes after shedding. Similarly, eggs were centrifuged at 33,000 *G,* removed and fixed immediately at 0° C. with Kahle's fixative. The eggs were fixed directly on coverslips, using the double coverslip sandwich technique, and then stained with various cytochemical dyes.

Black and white photographs were taken on Panatomic X film with a Micro-Ibso attachment (Leitz). Color photographs of fluorescence and dark field were recorded on high speed Ektachrome (daylight) with a Zeiss attachment camera with movable prism. When the latter film was used for dark field photographs, a No. 3 gelatin filter (Kodak) was also used in conjunction with an infrared filter to absorb the low blue illumination.

The author wishes to acknowledge the helpful assistance of Mr. Christopher Watters whose unbiased suggestions were most useful during this research.

Observations

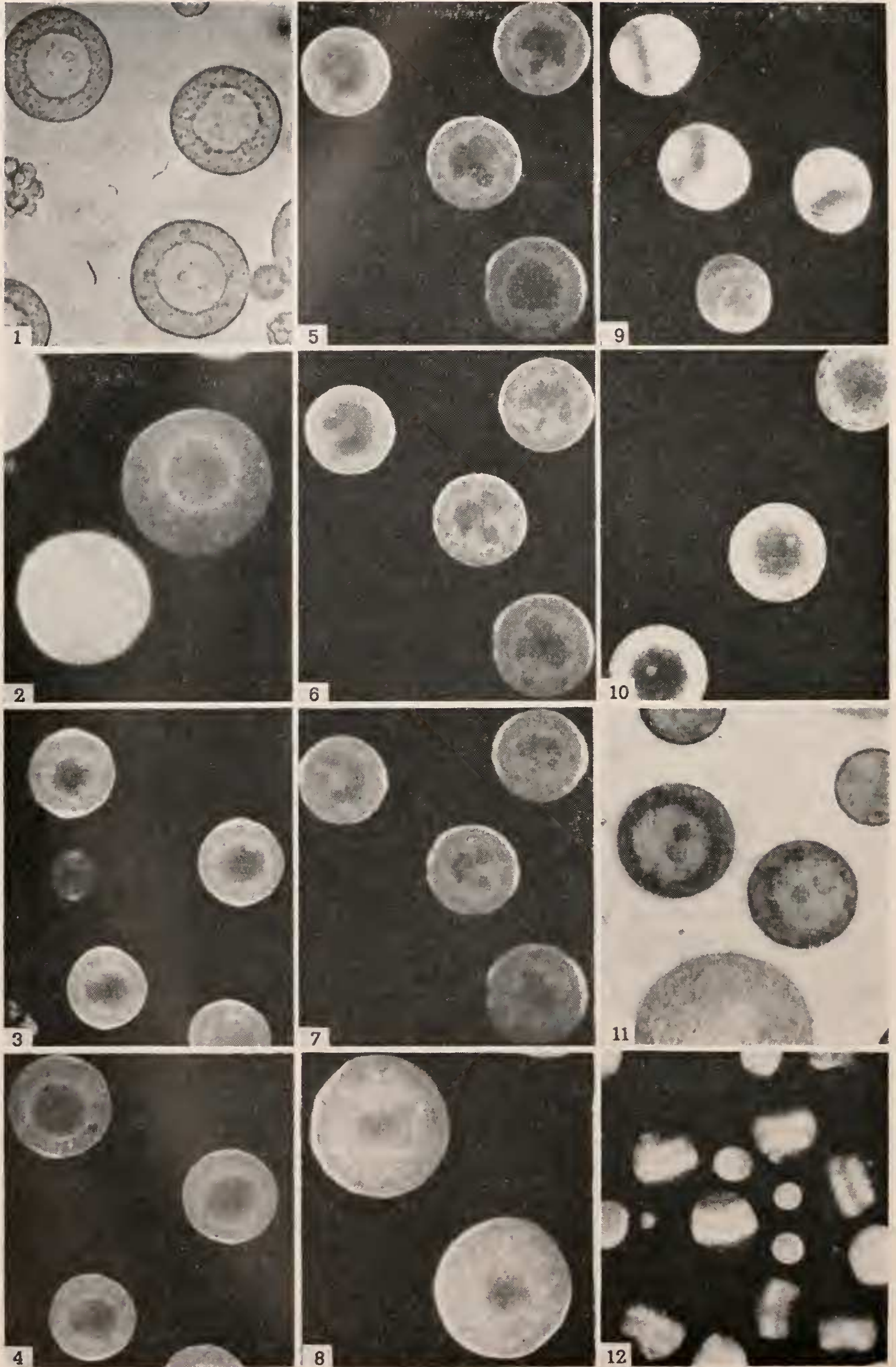
General appearance of the living egg

Prior to germinal vesicle breakdown the mature egg possesses a large germinal vesicle about two-thirds the size of the egg diameter. A prominent nucleolus, often double-lobed, is also present. The cytoplasmic ground color under visible light is pale yellow-green bounded by a yellow-green cell membrane. The egg measures 55 microns in diameter and is highly transparent although cytoplasmic particles are apparent (see Fig. 1).

Under dark field the particles are resolved into varying sizes of constantly agitated yellow-green granules. Smaller but extremely active silver-grey granules are more diffusely scattered throughout the cytoplasm. These also occur in irregular clumps around the nuclear membrane. The darker interior of the germinal vesicle contains a smaller number of similar granules. Sparsely scattered through the cortical cytoplasm are large yellow particles or clumps of granules.

Minute micro-villi project from the egg surface in a band covering the middle two-thirds of the egg. They are conspicuously absent in opposite polar regions and are much shorter in immature eggs. A living egg prior to germinal vesicle breakdown is seen under dark field in Figure 2.

Germinal vesicle breakdown. The eggs of *Pectinaria* at the time of shedding are slightly oval, flattened discs which almost invariably settle on their flattened surface. The germinal vesicle conforms to the oval shape but the outline is greatly scalloped during the first three minutes after shedding (Fig. 3). If the eggs are observed laterally, they resemble a dish standing on its edge. At 3.5 to four minutes post-shedding the germinal vesicle looses its scalloped edge and takes on a firm elliptical shape. Soon afterwards irregular clumps of highly motile, silver-white particles collect just outside of the membrane. The first indication of membrane breakdown is a wrinkling of the membrane that resembles the earlier scalloped appearance. Often, the nucleolus disappears at this time (Fig. 4). From six to ten minutes after shedding, depending upon the season, individual animals and temperature, the membrane opens, often on opposite sides. The silver particles flow through the gaps and form a temporary bridge, giving a



FIGURES 1–12.

transitory "hour glass" shape to the degenerating vesicle (Fig. 5). The initial breakthrough is followed by numerous irregular clumps of silver particles passing into the interior. The entire process of membrane dissolution lasts for 1.5 to three minutes (Figs. 6, 7). It is generally completed in all mature eggs 15 to 20 minutes after shedding. The nucleolus, if it has persisted, then gradually fades after membrane disappearance.

Soon afterwards, an indentation in the cell membrane forms on one side of the egg and beneath it a clear sac area develops. Here the first polar spindle forms

After disappearance of the germinal vesicle the egg has a small, dark irregular center surrounded by clumps of silver granules, numerous yellow granules of various sizes and points of fixed cortical granules (see Fig. 8).

Stratification of cell particles. Since dark field examination of the living egg had indicated the presence of several types of cytoplasmic particles, they were stratified by centrifugation. Living eggs were shed and allowed to undergo germinal vesicle breakdown. After cooling in an ice bath they were centrifuged for 15 minutes at 4000 G at 4° C. and examined immediately under dark field.

The cytoplasmic particles in *Pectinaria* consistently separated into three general regions, as seen in Figure 9. At the centripetal pole, an oval mass of fine, highly motile, silver white particles accumulated. This cap, referred to as zone A, consisted of small oil droplets at the apex and fine lipid particles.

FIGURE 1. Recently shed oocytes prior to GV breakdown. Unstained, reduced natural light.

FIGURE 2. A full size immature oocyte that did not undergo GV breakdown. Note the perinuclear ring of granules and the bilobed amphinuceolus. Dark field. 540 ×.

FIGURE 3. Newly shed (2.5 minutes) mature oocytes showing typical scalloped germinal vesicle. Dark field. 375 ×.

FIGURE 4. The same oocytes as in Figure 3 at 5.5 minutes after shedding. Germinal vesicle has become firm. 375 ×.

FIGURE 5. Mature oocytes just beginning GV breakdown at 9 minutes after shedding. The large, bright granular clumps are not involved. An amphiaster is forming on the extreme right. Dark field. 375 ×.

FIGURE 6. The same oocytes as in Figure 5 at 10 minutes after shedding. Notice the formation of cytoplasmic "bridges" across the old germinal vesicle. Dark field. 375 ×.

FIGURE 7. Germinal vesicle breakdown nearing completion at 12 minutes after shedding. Most of the silver white granules have flowed inward. Dark field. 375 ×.

FIGURE 8. Mature oocytes after germinal vesicle breakdown and 15 minutes after shedding. The oocytes remain in this condition until fertilization. Dark field. 540 ×.

FIGURE 9. Oocytes before and after GV breakdown that were centrifuged, showing stratification of cell particulates. In the post-breakdown egg is a crescent-shaped lipid zone (A), a hyaline zone (B), and a centrifugal granular zone (C). At the extreme centrifugal end is a flattened vacuolated zone. Dark field. 375 ×.

FIGURE 10. Fluorescing oocytes stained with acridine orange. The nucleolus is just disappearing at 10 minutes after shedding. Fluorescence: yellow, yellow green, and orange cytoplasmic granules; dark green GV; bright green nucleolus. 375 ×.

FIGURE 11. Immature oocytes stained with thionin demonstrating cytoplasmic granular uptake and slightly separated amphinucleoli, one staining reddish violet, the other remaining unstained. Mature oocytes are adjacent. Natural light. 750 ×.

FIGURE 12. Post-GV breakdown oocytes which were fluorochromed with acridine orange and centrifuged (15,000 G). The centripetal pole is up in most eggs (dark green fluorescence). The granular zone (C) consists of a centripetal bright yellow fluorescent band that is slightly separate from a more centrifugal band of yellow green and orange granules. The centrifugal pole fluoresces dark green. Filters: Corning 5113, 3486; Kodak 301. 375 ×.

Moving centrifugally a hyaline mid-region, designated as zone B, appeared black under dark field illumination. In the immature eggs, this area contained the germinal vesicle or nucleus of the oocyte. In this region also were scattered isolated clumps of fixed golden particles located in the cortical layer of the egg since they did not shift their position with centrifugation. These large golden, twinkling particles extended up into the silver lipid particles of the centripetal cap and later evidence showed that they reached to the extreme centrifugal end of the egg.

The lower centrifugal hemisphere of the egg, zone C, contained a heavy concentration of irregular sized yellow-green granules. Beginning near the equator of the centrifuged egg, where a diffuse layer of fine granules, the mitochondria, were detected, the granules of yolk increased in size and number toward the centrifugal end of the egg.

At the extreme centrifugal end of the egg, a vacuolated area containing a few dark granules was discernible.

The position of these stratified layers paralleled that found in the early investigations on *Chaetopterus* by Lillie (1906).

These three general zones were also seen in the immature and developing oocytes which still possessed a germinal vesicle. After centrifugation, a crescent-shaped cap of silver particles rested on top of the germinal vesicle at the centripetal pole. The germinal vesicle extended into the middle zone of the egg with the nucleolus displaced toward the centrifugal end of the vesicle. Sometimes a faint band of silver particles could be seen displaced halfway down within the vesicle or displaced around the nucleolus. Centrifugal to the germinal vesicle a U-shaped zone of mitochondria and yolk granules was found.

Staining the pre-breakdown egg

Earlier it had been found that living marine eggs could be fluorochromed with vital fluorescent dyes (Tweedell, 1959) and that the fluorescent inclusions persisted through subsequent cleavage and development. As a means of identifying these fluorescent cytoplasmic particulates, eggs of *Pectinaria* were freshly shed into a variety of vital fluorochromes so that the inclusions could be studied before GV breakdown. The general procedure for these dyes is outlined for acridine orange, a vital fluorochrome that produces metachromasia in living tissues. Acridine orange also has a specific affinity for DNA and RNA-proteins under controlled conditions (von Bertalanffy and Bickis, 1956; Tweedell, 1960a).

Eggs were shed into a 0.001% solution of acridine orange (CI 46005) in filtered sea water and allowed to stand for three minutes. They were then centrifuged lightly, the stain decanted off and the eggs washed twice in fresh filtered sea water to remove background fluorescence. This dye absorbs maximally at 4100 Å and thus most observations were made with a violet exciter filter that transmits maximally at 4100 Å and a yellow shade barrier filter plus an infrared filter. This combination gave a completely black background but provided green, yellow and red fluorescence. Substitution of a noviol "A" barrier filter allowed transmission of visible blue light that also permitted non-fluorescing components to be seen, a procedure useful when centrifuged eggs were viewed under dark field.

When eggs were fluorochromed as above, the cytoplasm fluoresced pale green

and was filled with numerous yellow-green and orange granules of various sizes; the entire egg was enclosed by an orange fluorescent cell membrane (Fig. 10). The light yellow-green nucleolus, containing one or more non-fluorescing vesicles, appeared within the dark green germinal vesicle, which was surrounded by a perinuclear band of yellow-green granules.

About seven minutes post-shedding, the nucleolus disappeared and distinctive bright green fluorescent clumps of a constant size and number, presumably chromosomes, appeared within the germinal vesicle.

Following GV breakdown, the egg cytoplasm contained a scattered mixture of yellow, orange and yellow-green granules. About 15 minutes after breakdown, the bright yellow-green chromosomes reappeared in a tight knot near the center of the egg in the first maturation metaphase. These two observations were the first ones seen of the chromosomes in the living egg of *Pectinaria*.

Further substantiation of the identity of the chromosomes before and after GV breakdown came from the application of histochemical dyes to the fixed eggs. The same assemblage of chromosomes could be seen in the intact egg following the Feulgen reaction, staining with gallocyanin chrome-alum under conditions made specific for nucleic acid concentration (Lagerstedt, 1949; de Boer and Sarnaker, 1956, cited in Pearse, 1961) and with Galigher's haematoxylin.

The chromosomes were also very sharply defined in the fixed whole egg after they were extracted with trichloroacetic acid and stained with Schiff's reagent.

Secondary fluorescence was also induced in the pre-breakdown egg with neutral acriflavine (National Aniline). Since this dye absorbs at the same wavelength as acridine orange, the same filters were used. Acriflavine has been shown to have an *in vivo* affinity for intranuclear proteins (De Bruyn *et al.*, 1953). A basic dye, it combines with the phosphoric acid groups of the nucleic acids (Brachet, 1957).

Eggs were shed directly into a 0.001% solution of the dye in sea water. In the living egg bright lime-green fluorescent granules were evenly dispersed through the cytoplasm. Within the nucleus, the nucleolus fluoresced bright yellow-green. Large vacuolated spheres were also seen within the nucleolus. With the exception of the nuclear membrane, the interior of the nucleus, strangely, remained unstained.

The nucleolus. The nucleolus in the mature oocyte was a large single body with one or more eccentrically placed vesicles or vacuoles, differentiated by their general lack of staining affinity. The body of the nucleolus fluoresced lime green with acridine orange or acriflavine, dark green with Janus green and blue with toluidine blue in the living egg. In fixed material, the nucleolus was Feulgen-positive and stained dark purple with gallocyanin chrome-alum. The vesicles or vacuoles remained unstained in each case.

In the younger oocytes the vesicles occurred as epinucleolar buds but in the mature oocytes, they appeared to be intranucleolar vacuoles (Raven, 1958). Both types existed. The immature oocytes that still possessed nucleoli after high speed centrifugation (33,000 *G*) occasionally showed a telescoped chain of three, sometimes four vesicles.

In the less mature oocytes, the nucleoli usually appeared as double-lobed amphinucleoli (Wilson, 1925), one lobe larger than the other. At times, the

nucleoli were separated but very often they were united. The principal nucleolus often had one or more accessory nucleolar buds attached to it.

The chemical differences within the amphinucleoli were apparent after staining in thionin, a basic vital dye that exhibits metachromasia. The smaller lobe generally stained deep reddish violet while the larger lobe remained perfectly clear (Fig. 11). In mature oocytes, the single nucleolus never became stained although this may have been a function of the short staining time before nucleolar breakdown.

The youngest oocytes, judged by their smaller diameters, generally possessed a greater number of small separate nucleolar-like bodies. No attempt was made to study the origin or development of these nucleolar bodies but it was noticed that the small nucleolar vesicles in the very young oocytes fluoresced bright red in contrast to the bright green of the main nucleolar body, after treatment with acridine orange.

Centrifugation of vitally dyed eggs

With the application of vital fluorochromes and other dyes to the pre-breakdown egg it became apparent that many granules of diverse shapes and sizes were scattered randomly throughout the cytoplasm. Many of these consisted of lipid and proteid yolk granules. However, even the common position of these which stained with different fluorochromes or dyes did not necessarily indicate that they were identical.

As a step toward resolving the mixture of cytoplasmic granules, the eggs were vitally dyed or fluorochromed after GV breakdown and centrifuged at 30,000 *G* for 15 minutes in a precooled head maintained at 4° C. throughout the run.

When the eggs fluorochromed in acridine orange were examined under blue light and a yellow shade filter, the principal fluorescence came from a heavy concentration of yellow or orange granules displaced toward the centrifugal pole in zone C. The rest of the egg appeared deep green (Fig. 12). The extreme centrifugal pole also contained an irregular agranular area that remained deep green.

In almost the exact center of the centrifuged egg, at the junction of the yolk granules and the empty mid-region, a tight knot of lime green chromosomes was often seen.

Substitution of a noviol A barrier filter revealed the silver blue cap of lipid granules in zone A. The deep green background of the hyaline zone changed to deep blue and the entire granular area in the centrifugal zone fluoresced violet-orange.

Proteid yolk and mitochondria. With more extensive centrifugation a second band of yellow-green granules was slightly separated from the main mass of orange yolk granules nearer the mid region of the egg. This distinct band appeared at various levels, depending upon the total centrifugal forces, but always rejoined with the main centrifugal mass of granules shortly after centrifugation. The fluorescence of these granules appeared to be identical to the perinuclear band of granules seen in the pre-breakdown egg.

After centrifugation the apparent homogeneity of the cytoplasmic granules stained with acriflavine also disappeared. A large concentration of yolk granules was again located in the heavy centrifugal end (zone C) of the egg. Under U. V. or blue violet light and a yellow barrier filter they fluoresced yellow green which became more intense nearer the upper end at the equator of the egg. However,

with blue violet illumination and a noviol filter that permitted blue violet transmission, these granules were quickly differentiated into a more centripetal band of brilliant, yellow fluorescent granules and a lower centrifugal zone of bluish granules. Fluorescence in the more centrifugal part of the zone was masked by the stronger transmitted blue light. Here was another indication that two general groups of granules made up the large zone at the centrifugal end of the egg (Fig. 13).

The majority of the granules concentrated in the centrifugal zone of the egg consisted of proteid yolk granules of different sizes. These granules were generally readily stained with several fluorochromes and other vital dyes. Centripetal to and overlapping the yolk granules in the equatorial zone of the egg was another layer of granules that were often differentiated by color and particularly on the basis of their fluorescence. The earlier cited centrifugation experiments of Harvey (1939) and Costello (1939; 1958) on the annelid egg indicated that the fluorescent band seen with acridine orange and acriflavine, just centripetal to the main mass of yolk granules in the egg of *Pectinaria,* corresponded to the position occupied by the mitochondria.

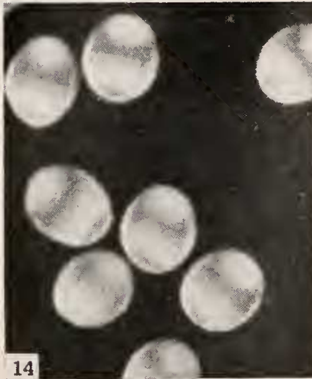
A separate yellow fluorescent equatorial band was also obtained after the application of two other distinctly different cytoplasmic fluorochromes, both used in the identification of fats (Popper, 1941). The first, thioflavine S (CI 49010) with a peak absorption at 3650 Å, was applied (0.0002%) to the living egg and observed under U. V. In the germinal vesicle stage the cytoplasm was filled with a mixture of yellow green granules. A separate perinuclear band of yellow green granules surrounded the pale green nucleus and a light green nucleolus.

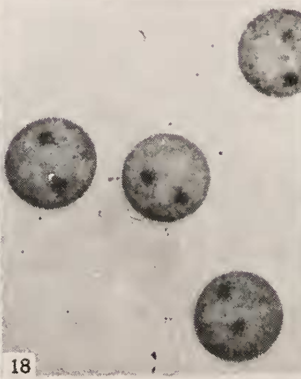
After centrifugation, both the U. V. and blue light illumination showed yellow green granules confined to zone C in the centrifugal pole of the egg. When U. V. light was coupled with a noviol O filter permitting full spectrum fluorescence, a separate yellow green band of granules could be identified across the upper end of the yolk granules in zone C. The greater mass of proteid yolk granules centrifugal to the band appeared blue since the complex formed by the dye and the yolk granules absorbed light at a higher wave-length.

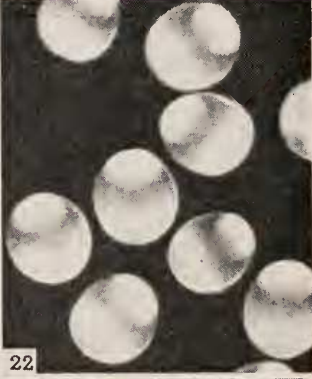
This distinction was not just the result of differences in intensity of fluorescence. A second fluorochrome, phosphine 3R (CI 46045), produced brilliant yellow fluorescence in a wide band of granules around the germinal vesicle. After GV disappearance, the vitally stained eggs were centrifuged and the whitish yellow granules were exclusively located in a thick band along the upper margin of the yolk mass in zone C. The rest of the granules did not fluoresce. This was the same area demarcated by acridine orange, acriflavine and thioflavine. It suggested that the difference in fluorescence was a qualitative measure of lipid content as well as particle size.

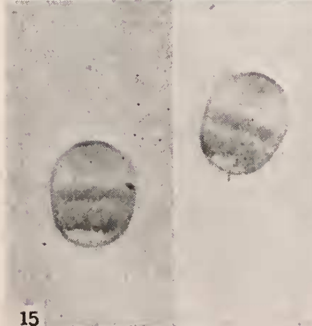
The lower centrifugal granular portion of zone C could also be sharply delineated by its fluorescence from the more centripetal fluorescent band with auramine O (CI 41000). This fluorochrome was very successfully used for dying the tubercle capsule (Richards and Miller, 1941) which is rich in neutral polysaccharides.

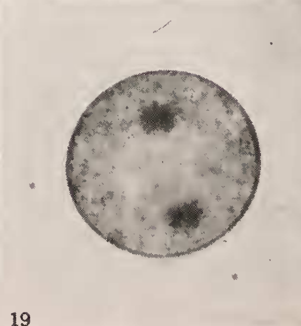
Application of a 0.001% solution of auramine O to the pre-breakdown egg produced fluorescence extending as a broad band of brilliant yellow cytoplasmic granules around the nuclear membrane.

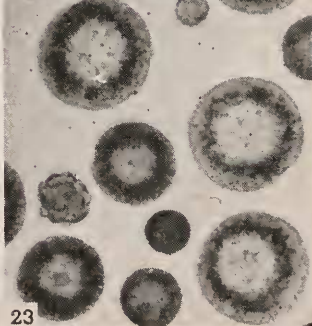


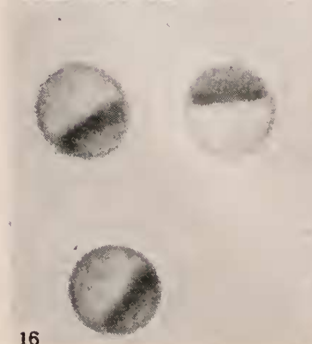












FIGURES 13–24.

Following centrifugation, the yellow fluorescent yolk granules were only found in the lower centrifugal region of zone C. The previous fluorescent band that fluoresced with acriflavine, thioflavine, and phosphine appeared blue from trans-illumination. Only the proteid yolk granules were activated by the U. V. illumination (Fig. 14).

The centripetal band of mitochondria in zone C was also clearly defined after staining with other vital dyes. Both Harvey (1941) and Monné (1944) found that gentian violet (crystal violet) gave a very intense stain with mitochondria in the sea urchin egg.

After staining the eggs with a 0.0001% solution of crystal violet (CI 42555) for 30 minutes and subsequent centrifugation at 33,000 *G* for 15 minutes, a sharp blue band of granules appeared at the centripetal end of zone C. This corresponded in position to the band seen previously with the fluorescent dyes (Fig. 15).

A temporary light blue ring was also seen just below the lipid cap in zone A but this diffused rapidly into the lipid cap.

Mature eggs of *Pectinaria* were next shed into another vital thiazine dye,

FIGURE 13. Post-GV-breakdown oocytes that were fluorochromed with acriflavine and centrifuged. The centripetal pole (upper left) contains blue illuminated granules. The lower centrifugal pole has a bright yellow band of fluorescent granules above the more centrifugal bluish-yellow granules. Filters: Corning 5113, 3060; Kodak 301. 375 ×.

FIGURE 14. Centrifuged oocytes that were fluorochromed with auramine and centrifuged. The oval centripetal zone appears blue. In the centrifugal zone only the lower proteid yolk granules fluoresce yellow. Filters: Corning 5850, 3389; Kodak 301. 375 ×.

FIGURE 15. Centrifuged oocytes stained with crystal violet. The lipid cap at the centripetal pole is surrounded by a band of light blue granules. Across the center is a blue violet granular band, slightly separated from the centrifugal yolk granules. 375 ×.

FIGURE 16. Centrifuged oocytes stained with Janus green. An intense layer of deep green granules lies just below the equator. More diffuse grey green granules extend to the centrifugal pole. 375 ×.

FIGURE 17. Centrifuged oocytes stained with Nile blue sulphate. The wide mass of dark blue granules at the centrifugal pole is slightly separated from an equatorial band of metachromatic (reddish-purple) granules. 375 ×.

FIGURES 18, 19. Post-GV-breakdown oocytes that were stained with Nile blue sulphate, showing metachromatic astral granules. Deep blue yolk granules are scattered through the blue cytoplasm. Natural light. Figure 18 shows eggs after recovery from centrifugation. 375 ×. Figure 19 is an uncentrifuged egg. 860 ×.

FIGURE 20. Centrifuged oocytes after GV breakdown. These eggs were fixed and stained by the Nile blue test. A deep red lipid drop and red violet lipid cap occur at the centripetal end. The hyaline layer is light blue. At the equator is a band of metachromatic granules and scattered purple granules. The centripetal zone contains deep purple granules. 500 ×.

FIGURE 21. Centrifuged eggs after GV breakdown, fixed and stained with Sudan black B. Black lipid droplets and a lipid granular cap located the centripetal end. A faint granular ring surrounds the lipid cap and a broad band of granules in zone C lies centripetal to the centrifugal pole. The immature oocyte on the right shows similar stratification plus a thin granular zone within the germinal vesicle. 500 ×.

FIGURE 22. Post-GV-breakdown oocytes stained with rhodamin O and centrifuged. The pink band of granules centripetal to the lipid cap is faintly detectable. Bright yellow green granules fill the centrifugal pole. Dark field. 375 ×.

FIGURE 23. Pre-breakdown oocytes stained with toluidine blue O. A heavy concentration of deep blue granules surrounds the nuclear membrane and grades out into the light blue cytoplasm. 465 ×.

FIGURE 24. Post-GV-breakdown oocytes stained with toluidine blue and centrifuged. A heavy collection of blue granules occurs at the centrifugal end. Just centripetal to the unstained lipid cap is a thin band of metachromatic granules. The hyaline layer is faint pink. 375 ×.

thionin (CI 52000), also used by Harvey (1941) for the sea urchin egg. This dye penetrated the living egg extremely slowly at all concentrations but did produce a faint lavender band of particles just outside the nuclear membrane. During GV breakdown, these particles collected along the indentations of the degenerating membrane. The particles became diffusely scattered throughout the cytoplasm after GV breakdown. Centrifugation failed to demonstrate any localized band of particles.

The immature eggs took up the stain readily, which first concentrated in the perinuclear granules and the nucleoplasm. As mentioned, the most effective concentration of the dye occurred in the nucleoli (see Fig. 11).

To further identify and localize the mitochondria, Janus green B (CI 11050) was applied to the living eggs. The specificity of Janus green for mitochondria has been well substantiated in a variety of marine eggs. For example, centrifugation experiments using Janus green as an index of mitochondria in eggs of *Ciona intestinalis* and *Phallusia mamillata* by Mancuso (1959), and La Spina (1958) all showed the mitochondria localized in the centrifugal half of the egg between the yolk and the centrifugal hyaline cap.

After eggs had been shed into a 0.001% solution of Janus green in sea water, diffusely scattered granules with a rather faint blue green coloration appeared throughout the cytoplasm. In immature eggs, there was a tendency for these granules to clump around the nuclear membrane in a perinuclear ring. After GV breakdown, they remained diffusely scattered in the cytoplasm.

Centrifugation of the eggs stained with Janus green generally showed that the blue green granules were confined to a slightly concave area in the centrifugal end of the egg. Rapid cross-comparison of the centrifuged egg under dark field with the stained egg under bright field demonstrated that the concentration of Janus green particles overlapped the heavy granular centrifugal portion of the egg. The cortex around the granular area remained unstained and well defined. In addition, strongly defined cortical granules were often seen to extend around the circumference of the egg.

The position of the displaced granules was not always consistent in the centrifuged egg. Often, a sharp, broad band of granules was localized across the equator of the egg while more grey green granules extended toward the centrifugal end. This heavy equatorial band always appeared in the centripetal portion of zone C, and in these cases corresponded to the position where the mitochondria usually stratified (Fig. 16).

Shaver (1957) found that Janus green was not satisfactory for demonstrating mitochondria in the intact cells of sea urchin embryos. Under phase contrast he observed they had a tendency to clump, indicating that a physical change took place in the granular elements after dye penetration. A similar phenomenon may have caused the inconsistent localization of mitochondria by Janus green in the living egg of *Pectinaria* after centrifugation.

The previous tests that differentiated the mitochondria from the proteid yolk suggested the use of Nile blue sulphate on the living eggs. Nile blue sulphate (CI 51180) is an oxazine dye used for histochemical differentiation of lipids. It is a commonly used vital dye and exhibits metachromatic properties even in the living egg. Monné (1944) had found that Nile blue stained the yolk platelets blue in the sea urchin egg. However, blastulae of the sea urchin were vitally

stained with Nile blue sulphate by Gustafson and Lenique (1952) which enabled them to follow the number and distribution of mitochondria.

The eggs of *Pectinaria* were shed into a 0.001% solution and subsequent to GV breakdown, the cytoplasm gradually became light blue and the yolk granules became deep blue. In the immature eggs in which the nucleus had not broken down, an elliptical band of heavy purple granules eventually appeared around the intact nuclear membrane.

After centrifugation at 4160 *G* for 15 minutes, the post-breakdown egg showed a heavy concentration of deep blue granules at the centrifugal end. Toward the equatorial region of the egg, the granules of zone C formed a distinct band of reddish purple granules. This band occupied the same position as the band of granules seen with the previous fluorochromes. Under prolonged centrifugation at 33,000 *G* the band was distinctly separated from the yolk and remained in the mid region of the egg. The centripetal cap in zone A remained unstained (see Fig. 17).

Astral granules. In the mature egg the metachromasia induced by Nile blue sulphate did not become evident until after germinal vesicle breakdown and the formation of the first maturation spindle. Upon standing in fresh sea water after staining, many of the eggs exhibited two crescent-shaped groups of granules that accumulated around each end of the clear spindle area near the equator of the egg. These constantly agitated granules appeared about 45 minutes after shedding. Under visible light they appeared reddish violet, in sharp contrast to the uniform blue stained yolk granules. Their uniform position in all the eggs (Figs. 18, 19) suggested that they were associated with the astral rays at each end of the maturation spindle.

The same phenomenon was observed in the centrifuged eggs after the stratified granules had begun to return to their original position. Within three hours after centrifugation, identical groups of reddish violet granules were found in these eggs. In many instances they were grouped around the first polar body which often formed after centrifugation.

Extended observations of these eggs revealed that the astral granules originated from the equatorial band of reddish granules in zone C.

Nile blue test. Fixed centrifuged eggs were next submitted to the Nile blue histochemical test (Casselman, 1959) in order to verify the metachromatic staining seen in the living egg. The granules in the lower part of zone C consistently stained deep blue, the identical result found in the living centrifuged eggs. It also suggested that these granules were primarily proteid yolk since the oxazine component of Nile blue forms blue salts (Cain, 1947, cited in Casselman, 1959) with fatty acids and phospholipids. The more centripetal band of zone C was again filled with reddish purple granules. A non-acidic red reaction is created by the oxazone component with glycolipids and simple lipids. It seemed likely that these granules were rich in glycolipids.

A strong reddish purple reaction was also produced in the centripetal cap of zone A. Just above this cap, at the extreme centripetal end of the egg, one or more wine red lipid droplets collected after centrifugation, just within the cell membrane. Very often they coalesced into a single red lipid droplet. Scattered between the lipid cap in zone A and the centrifugal zone were sparsely scattered blue granules, apparently located in the cortex (see Fig. 20).

It is well established that fatty substances are found in oocytes of many different animals (Raven, 1958, 1961). These may occur as free lipids in the cytoplasm, as fatty yolk globules or they may be contained within cell components such as the mitochondria, Golgi bodies, etc.

One of the best cytochemical indicators of overall lipid distribution is Sudan black B. Since the dye is actually dissolved in the lipid, but not in water, it must be used on fixed material.

Centrifuged eggs were fixed in 10% formalin and 1% $CaCl_2$; the eggs were dyed with a 1% solution of Sudan black B in 60% triethyl phosphate (Casselman, 1959) and mounted in glycerin jelly (see Fig. 21).

At the centripetal end of the egg in zone A, the lipid cap stained black and was surrounded centrifugally by a light grey band of fine granules. Either within the lipid cap or just centripetal to it were one or two large intensely black droplets formed by free lipids.

The hyaline zone was clear except for prominent black isolated cortical granules extending the full length of the stratified eggs.

Moving centrifugally, a prominent band of dark granules surrounded the equator of the egg. The position of this band was identical to the mitochondrial band previously delineated by the fluorochromes and vital dyes. Very often a clear indentation in the band indicated the presence of the maturation spindle.

The rest of the centrifugal zone consisted of lighter stained diffusely scattered granules. At the extreme centrifugal end of the egg, another empty, vacuolated area appeared overlaid by the prominent peripherally located black cortical granules.

The disposition of the mitochondria in the centrifuged egg was found to coincide with many of the granules that consistently fluoresced with lipophilic dyes (Richards, 1955; Metcalf and Patton, 1944). However, not all of the dyes showed that the mitochondria were concentrated in the equatorial zone of the centrifuged egg. This was the case after staining with rhodamin O (CI 45170), a vital dye and fluorochrome that has been used in the identification of mitochondria (Lillie, 1948).

Under U. V. illumination, eggs shed and dyed in a 0.001% solution of rhodamin in sea water revealed small, yellow orange particles evenly distributed throughout the cytoplasm. These same particles under dark field illumination appeared brilliant pink and had the additional advantage of being sharply differentiated from the natural luminescence of the cytoplasmic particles seen under dark field.

After staining, eggs were centrifuged at 33,000 *G* for 15 minutes in a pre-cooled head. The pinkish particles always formed a crescent-shaped band around the centripetal end of the germinal vesicle in the immature eggs.

In the mature eggs after GV breakdown, the pink particles were concentrated in a ring that was a part of the lipid cap in zone A. This ring formed a border around the centripetal edge of the cap (Fig. 22).

The appearance of the pink peripheral band around the lipid cap indicated that certain of the granules were distinctive from the general mass. This was also suggested from the results after Sudan black application to fixed centrifuged eggs which showed a thick peripheral band around the lipid cap. The specificity of rhodamin O for these particles strongly suggested that they represented a second type of mitochondria.

The ring was transitory in its appearance. A few minutes after the stratified eggs were removed from the cold, the pink particles of the band would move into the

center of the lipid cap. Here they would form a diffuse oval of pink particles within the center of the silver white particles.

A similar centripetal band of granules lying around the periphery of the lipid cap was detected after fluorochroming with thioflavine.

Post-breakdown eggs were shed into a 0.0001% solution of thioflavine in sea water, stained for 10 minutes and centrifuged at 33,000 *G* for 15 minutes. When the preparations were excited with U. V. light (filters: 9863, 5970 or 18A) and observed with a noviol O filter, a definite yellow green band appeared centripetal to the blue violet appearing lipid cap. This band occurred in a position identical to that taken by the pink granules seen with rhodamin O.

An identical band of granules was demonstrated in living eggs that were dyed with toluidine blue. Eggs were freshly shed into 0.0001% solutions of toluidine blue (CI 52040) in sea water. Within ten minutes a heavy concentration of dark blue granules appeared around the germinal vesicle. This band graded off into smaller sparsely scattered blue granules throughout the rest of the cytoplasm. Around the periphery of the cell, just inside the cell membrane, a single row of dark blue granules was also found (Fig. 23).

After GV breakdown the blue granules were dispersed toward the periphery of the egg in the outer two-thirds of the cytoplasm. Upon prolonged staining in a dilute solution of the dye, smaller red violet metachromatic granules became apparent in the post-breakdown eggs. These were evenly distributed throughout the cytoplasm.

The dark blue granules were easily concentrated into the centrifugal hemisphere of the egg after centrifugation for 15 minutes at 4000 *G*. Here, the heavier yolk granules and the more centripetal zone of mitochondria all stained the same. The dark blue granules then graded off into the equatorial zone of the egg. The location of the metachromatic granules was clearly defined in the more heavily centrifuged eggs. Just centripetal to the clear yellow lipid cap in zone A, a ring of reddish purple metachromatic granules became concentrated (see Fig. 24). This sharply defined band was easily seen with natural light and appeared to be identical to the similar band of pink particles seen with rhodamin O under dark field illumination.

Since the metachromasia of these particles somewhat resembled that seen after eggs were stained with Nile blue sulphate, post-breakdown eggs were stained with each dye and centrifuged concurrently. The position of the metachromatic granules caused by toluidine blue was always quite distinct from those produced after application of Nile blue sulphate.

The hyaline region

Relatively few granules were seen in the hyaline region after centrifugation except those that drifted in from either of the adjacent stratified layers. The cytoplasm did exhibit a deep green fluorescence with acridine orange and acriflavine, thus suggesting a high nucleic acid concentration. Photographs of the centrifuged sea urchin egg taken with ultraviolet light showed the greatest absorption in the hyaline layer, thus indicating nucleic acid compounds in this layer (Harvey and Lavin, 1944). Similarly, the clear area stained pinkish blue with toluidine blue and pink with rhodamine B.

In the fixed egg, Nile blue sulphate colored the area light blue and Schiff's reagent in the plasmal test gave it a bright pink-violet color.

The presence of extremely small particles, rich in nucleic acid, in this hyaline area was indicated after the application of gallocyanin chrome-alum. The lower two-thirds of the hyaline zone contained a diffuse collection of dark blue particles.

The centrifugal pole region

This irregular hyaline area, just centrifugal to the yolk mass, constituted an enigma because of the apparent lack of particles present. With fluorescent dyes, it gave the same fluorescence as seen in the more centripetal hyaline band. None of the vital dyes indicated the presence of any granules.

However, in the Nile blue test on fixed eggs, this area appeared to be formed from a heap of irregular vesicles, each having dark blue granules around their borders. On the assumption that this area represented a structural phase of the cytoplasm, fixed centrifuged eggs were stained with gallocyanin chrome-alum at pH 0.8. The results indicated that a cone of very dark blue-black basophilic granules occupied the centrifugal pole, probably embedded in the structural phase of the cytoplasm.

Discussion and Conclusions

Each of the vital dyes and fluorochromes, when combined with centrifugation, provided specific information about one or more cellular inclusions in the oocytes of *Pectinaria*. From these results a composite picture of these inclusions in the centrifuged cell was projected, based upon their specific staining properties and stratification pattern.

In general, these investigations showed a stratification pattern as follows, moving from the centripetal to the centrifugal end of the egg: (1) oil droplets, (2) a cap of lipid granules, (3) a diffuse layer of fine granules, (4) a hyaline zone with the germinal vesicle (in immature eggs), (5) a broad layer of heavier granules, (6) a zone of heavy yolk spheres, (7) an irregular vacuolated area with scattered basophilic granules. Throughout the cortical region of the entire centrifuged egg were isolated clumps of cortical granules. A composite diagram of the centrifuged oocyte after germinal vesicle breakdown is shown in Figure 25.

The first inclusions at the centripetal pole were oil droplets that often fused into one or two large drops under prolonged centrifugation. Sudan black B turned them intensely black. These oil drops also stained bright red or pink when the Nile blue test was applied to the centrifuged fixed egg, which indicated the presence of neutral lipids.

The centripetal lipid granular cap reacted similarly. Sudan black formed a black cap, Nile blue sulphate turned it reddish violet in fixed eggs, and it stained an intense violet with Schiff's reagent in the plasmal test.

While the lipid cap could be easily seen under dark field or blue violet illumination, few of the fluorochromes used induced fluorescence in the lipid granules. An exception was thioflavine which induced yellow green fluorescence in the lipid cap.

The granular distribution found by Harvey (1941) in the sea urchin egg and in the annelid egg (1939) following centrifugation showed a mitochondrial band near the mid-region of the egg, just centripetal to the yolk mass. Monné (1944) and Monné and Hårde (1951) noted that centrifugation of the living sea urchin egg

after staining with gentian violet or methylene blue resulted in the mitochondria concentrating at the extreme centrifugal end of the egg.

However, electron microscope studies of stratified sea urchin eggs by Lansing, Hillier and Rosenthal (1952) indicated that two layers of mitochondria stratified, one of high density just above the yolk granule layer and one of low density in the centripetal lipid layer.

In similar studies, Gross, Philpott and Nass (1956) reported that the mitochondria were concentrated in a layer centripetal to the yolk, yet they concluded

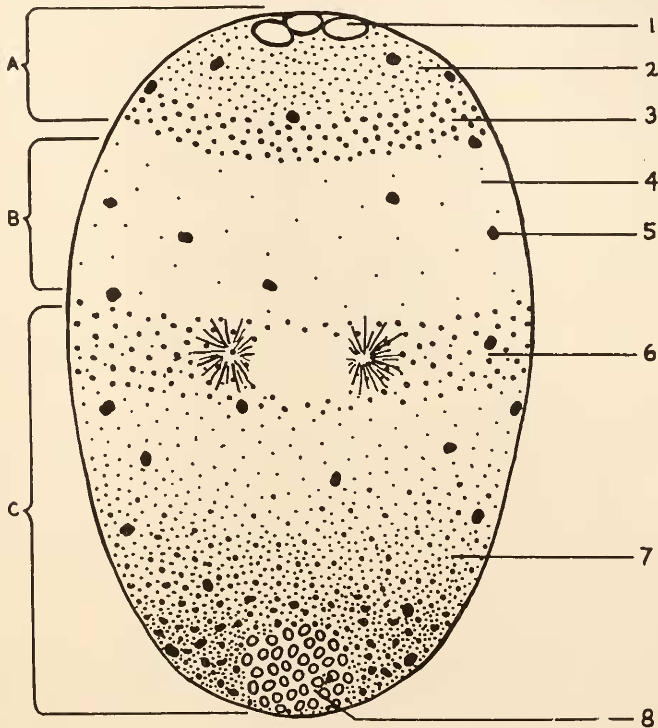


FIGURE 25. A composite diagram of the centrifuged oocyte of *Pectinaria* after GV breakdown. The stratified particles were stained by different vital dyes, fluorochromes and cytochemical tests. The three zones of the unstained centrifuged egg (A, B and C) are indicated, beginning with zone A at the centripetal pole. 1, Fat droplets. 2, Lipid granular cap. 3, Granular band I. 4, Hyaline zone with diffuse granules. 5, Cortical granules. 6, Granular band II, mitochondria and proteid yolk. 7, Dense proteid yolk. 8, Centrifugal vacuole with basophilic granules.

that the mitochondria move to new positions both above and below the clear zone (zone B). Shaver (1957) also reported particles likened to mitochondria lying in an area just beneath the lipid cap (zone A).

The relative distribution of particles identified as mitochondria in ascidian eggs seems to follow the same pattern. Reverberi (1956; 1957b) believes there are probably at least two kinds of mitochondria; one type is osmiophilic, does not stain

with Janus green and localizes near the centripetal pole. The second type gathers above the equator and stains with Janus green. The position of the latter, however, conflicts with the finding of Mancuso (1959) and La Spina (1958) in eggs of *Phallusia*.

This description agrees with the evidence presented here of a second mitochondrial band existing just below the lipid cap in the centrifuged eggs of *Pectinaria*. The centripetal mitochondrial band is distinctive from the centrifugal band since it selectively stains bright pink with rhodamin O. Moreover, an identical yellow green fluorescent band centripetal to a blue lipid cap is also seen with thioflavine. Both of these acid fluorochromes are lipophilic. The lighter band of granules does not stain with Janus green B.

It should be noted, however, that a similarly transitory light blue band does form with crystal violet. Harvey (1941) found that the latter stained the centrifugal mitochondrial band purple (metachromatically) in the sea urchin egg.

A distinct band in the same position, just centripetal to the lipid cap and separated by a thin, clear band, was also detected following application of Schiff's reagent in the plasmal reaction.

More significantly, the centripetal band in the egg of *Pectinaria* reacted selectively with toluidine blue, forming a characteristic metachromatic reddish-violet band of granules just under the lipid cap.

Toluidine blue, a cationic dye, stains basophilic substances blue and reacts metachromatically with certain chromotropes as a result of polymerization of the basic dye (Michaelis, 1947).

The chemical interpretation of metachromasia is very tenuous without parallel confirmatory tests and controlled reactions. However, the substances that the metachromatic reaction detects are high molecular weight moieties with free anionic groups. This includes anionic mucopolysaccharides, both DNA and RNA nucleic acids and some anionic lipids capable of polymerization (Schubert and Hamerman, 1956). Some substances that cause metachromasia are heparin, chrondroitin sulphate and hyaluronate.

In a survey of metachromasia with toluidine blue, Kelley (1954) found metachromasia in eggs and ovarian tissue of many animal species, including *Arbacia, Chaetopterus* and *Spisula*. Metachromasia generally occurred in the cytoplasm and in the jelly around the eggs.

Metachromasia with toluidine blue was also noted by Dalcq (1957) in a study of the ascidian egg and in the developing rat egg (Dalcq, 1954) where he found a correlation between the localization of metachromatic granules and distribution of mucopolysaccharides.

Similar metachromasia occurs *in vivo* in the molluscan eggs of *Barnea* and *Gryphaea*, and in the sea urchins, *Psammechinus miliaris* (Pasteels and Mulnard, 1957) and *Paracentrotus lividus* (Pasteels, 1958). In these developing eggs stained with toluidine blue, they note very fine, blue granules (alpha granules) which are uniformly distributed throughout the cytoplasm. Upon centrifugation, these granules accumulate at the centrifugal pole. At the appearance of the sperm aster, new granules (beta granules) appear which are strongly metachromatic. The beta granules are believed to derive their metachromasia from the blue alpha granules when they become associated with the astral rays. With

strong centrifugation, the beta granules are stratified beneath the lipid cap. From this and other evidence of acid phosphatase activity and acid mucopolysaccharides, Pasteels and Mulnard identify the beta granules as mitochondria.

These metachromatic granules are thus like those seen in *Pectinaria* in two respects, their reaction with toluidine blue and their position in the stratified egg. However, the centripetal band of metachromatic granules in *Pectinaria* is not seen associated with the astral rays.

Mulnard (1958) reported identical metachromatic α-granules and β-granules in the eggs of *Chaetopterus pergamentaceus* after they were stained with brilliant cresyl blue. He also described the presence of a third granule, material X, which he believed was the precursor of the β-granules.

The oocytes of *Spisula solidissima* were vitally dyed with methylene blue and toluidine blue by Rebhun (1959; 1960). Eggs left standing in dilute concentrations of either dye contained metachromatic granules which were extensively studied and analyzed. Rebhun found two sets of metachromatic particles, both directly stainable with toluidine blue. These particles measured ¼ to ½ micron in diameter initially and were uniformly distributed through the cytoplasm.

At the time of spindle formation, during the formation of the polar bodies or in the subsequent cleavages, Rebhun found that the metachromatic granules became associated with the aster. Both sets of granules migrated directly into the aster. This unusual behavior will be discussed in connection with Nile blue sulphate.

When the oocytes were centrifuged before GV breakdown (8000 *G* for 1½ to 4 minutes), the granules were mainly located in a narrow layer centrifugal to the germinal vesicle. A few were found in the lipid cap and in the centrifugal yolk area.

After GV breakdown, centrifugation stratified the metachromatic granules in two locations, a layer at the centripetal end of the hyaline zone and a layer at the centrifugal end of the yolk area.

Rebhun equates the centripetal particles with the β-granules of Pasteels (1958) on the basis of their location in the centrifuged egg and their migration into the asters. He also identifies the centrifugal particles with the α-granules of Pasteels *et al.* but these are not astrally located according to Pasteels and Mulnard (1957).

On the basis of their metachromatic staining and stratification in the centrifuged egg, the centripetal granules in the oocyte of *Pectinaria* appear to be very similar to the centripetal layer of metachromatic granules that Rebhun finds in *Spisula*. However, the failure to see the metachromatic granules in *Pectinaria* associated with the asters during the first polar body formation cannot be explained from the present findings. It is possible that they were not detected or that they do not appear until after fertilization.

Demonstration of the proteid yolk particles in the living egg was easily accomplished even though the dyes were not always specific. Proteid yolk fluorescence was orange with acridine orange, yellow green with thioflavine and yellow with acriflavine. These granules were readily vitally stained pink with neutral red, deep blue with toluidine blue or Nile blue sulphate.

The reaction of similar dyes in the sea urchin led Monné (1944) to suggest that the yolk is composed of a phosphoprotein combined with lipids. Later, dif-

ferential staining of various polysaccharides caused Monné and Slautterback (1950) to conclude that the yolk probably consisted of aminopolysaccharide combined with a protein and lipid.

Raven (1958) subdivides the yolk granules of *Limnaea* into fatty yolk, consisting of free lipids and fat globules, and proteid yolk. The latter, composed of two granular types, is rich in mucopolysaccharides.

The specificity of auramine for the proteid yolk granules in the eggs of *Pectinaria* indicates the presence of mucopolysaccharides, probably in loose combination with proteins.

Just centrifugal to the hyaline layer in the pre-and post-GV-breakdown egg of *Pectinaria*, an equatorial group of granules is concentrated into a band just centripetal to and overlapping with the yolk granules. The identification of a similar band in centrifuged eggs of the sea urchin (Harvey, 1939, 1941, 1944) and in the eggs of *Chaetopterus* (1939) and *Nereis* (Costello, 1939, 1958) strongly suggests that the band is rich in mitochondria.

Differential fluorescence of this granular band was produced with thioflavine, acridine orange, acriflavine and phosphine. Crystal violet also selectively stained it. Less specific but positive identification was obtained with Janus green and Sudan black B.

In the sea urchin egg Harvey (1941) was able to stain the mitochondria with both gentian violet and Janus green. Monné (1944) also reported mitochondrial staining with gentian violet.

The specific staining of similar stratified granules in *Pectinaria* by Janus green and gentian violet suggests the presence of mitochondria in this band.

Induced fluorescence of granules in the same stratified position by acridine orange, acriflavine, thioflavine and phosphine suggests that these fluorochromes are also staining the mitochondria. In particular, thioflavine, which is lipophilic, also induces fluorescence in the mitochondrial band just centrifugal to the lipid cap.

The possibility still exists that the induced fluorescence is caused by similarly localized but not identical granules since the mitochondria do overlap with the yolk granules. In the centrifuged eggs of *Limnaea stagnalis,* Raven (1958) also finds a mixture of mitochondria and γ-granules, one type of proteid yolk, in an analogous position. While the fluorescence of this band of granules with the above fluorochromes under the described conditions appears to be specific, the exact nature of the granules fluorescing requires further verification.

In the living eggs of *Pectinaria* Nile blue sulphate produced striking metachromasia of granules that localized in a band corresponding to the position of the mitochondria. This was also confirmed in whole fixed centrifuged eggs. The metachromasia produced indicated the granules contained glycolipids.

In *Pectinaria* the astral granules also have their origin in the metachromatic granules produced by Nile blue sulphate. It would be tempting to assume that the metachromatic astral granules were mitochondria. Gustafson and Lenicque (1952) used Nile blue sulphate to follow the mitochondria in the developing sea urchin egg. They postulated that the stain adhered to the lipid-rich sheath that surrounds the mitochondrium. However, they did not report that the granules were metachromatic.

Raven (1958) indicated that the mitochondria in *Limnaea stagnalis* gather around the maturation spindle and often migrated in between the astral rays. He also noted that the aster was surrounded by the centripetal γ-granules that were distinct from the centrifugal proteid yolk granules.

Except for their stratified position in the mitochondrial layer, there is not too much evidence to indicate that the metachromatic astral granules in *Pectinaria* are mitochondria. They also differ from the granules stained with mitochondrial dyes and fluorochromes in that the astral granules are only seen with Nile blue sulphate.

In addition to the metachromatic astral granules in *Pectinaria,* similar granules have been seen in related eggs by others.

Taylor (1931) described natural red granules dispersed through the cytoplasm of the echiuroid, *Urechis caupo.* In the centrifuged egg they gathered in clusters at the extreme centrifugal end of the egg. When the amphiaster formed during maturation and cleavage, in the normal or centrifuged egg, the granules migrated along the astral rays and finally surrounded the nucleus of each daughter cell.

Iida (1942) also followed particles stained with neutral red along the astral rays and along the mitotic spindle in the sea urchin egg. These granules were also distributed to the daughter cells.

In this connection, Lillie (1906) observed that neutral-red-stained granules collected in a ring around the first maturation spindle in *Chaetopterus* oocytes. He indicated that they were derived from granules composing the "residual substance" or the germinal vesicle.

It is fairly certain that this is not the case in oocytes of *Pectinaria.* However, a thin layer of granules, perhaps equivalent to the residual substance, does stratify *within* the germinal vesicle. It is possible that the latter granules are those which layer just centrifugal to the lipid cap after GV breakdown in *Pectinaria* (see Fig. 26).

Vitally stained granules were observed by Kojima (1959a, 1959b) in several sea urchin eggs and in the egg of *Urechis unicinctus.* In the fertilized eggs that were stained with neutral red, toluidine blue, Janus green or Nile blue sulphate, deeply stained granules appeared around the aster. In the unfertilized egg the granules collected around the germinal vesicle. After strong centrifugation, the granules displaced into the centrifugal pole of the egg.

It was difficult to equate the metachromatic astral granules of *Pectinaria* with these varied findings. First, the nature of the granules was not clearly established. The neutral-red-staining granules seen by Lillie were obviously different. Those reported by Iida and Kojima were not observed in *Pectinaria.* The data of Kojima for Nile blue sulphate and toluidine blue indicated different levels of stratification and lack of metachromasia.

In *Spisula* oocytes Rebhun (1959) found two types of metachromatic particles, the centripetal β-particles already referred to and the α-particles which layered in the centrifugal hemisphere. Both types migrated into the asters and each was excluded as being mitochondria.

It is not likely that the metachromatic granules produced by Nile blue sulphate in *Pectinaria* can be identified with the α-particles seen with toluidine blue in *Spisula.* First, the two dyes in question stain different particles in the eggs of *Pectinaria.* Secondly, the two sets of particles stratify in different layers upon

centrifugation, the astral granules being derived from a layer that is coincident with the mitochondria in *Pectinaria*. This is not the case in *Spisula*.

In electron microscope studies, Rebhun (1960) identified the metachromatic granules as multivesicular bodies and concluded that the metachromatic granules were definitely not lipid, mitochondrial, yolk or cortical granules. Two other components were present, the Golgi bodies, particularly plentiful in early oocytes, and annulate lamellae which occasionally orientated with the asters. It is possible that one of these particles is analogous to the astral granules of *Pectinaria*.

The nucleolus of the mature oocyte of *Pectinaria*, characteristically an amphinucleolus, was strongly basophilic in the cortical region but the nucleolar vacuoles remained unstained (Fig. 26). All of the vital dyes and fluorochromes applied to the oocytes indicated that the body of the amphinucleolus was principally composed of DNA. This was confirmed after treatment of the fixed eggs with the Feulgen

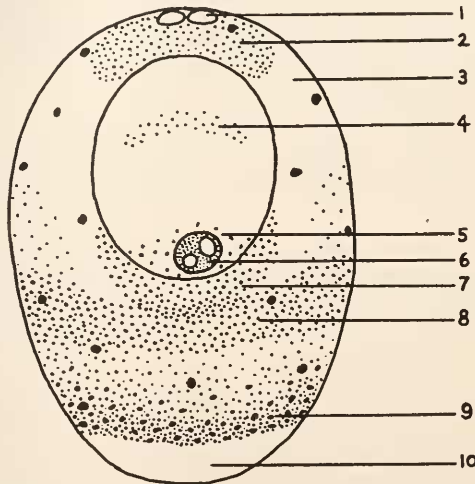


FIGURE 26. A centrifuged immature oocyte, showing particle distribution in relation to the intact germinal vesicle. 1, Fat droplets. 2, Lipid granular cap. 3, Hyaline zone. 4, Germinal vesicle substance. 5, Basophilia. 6, Amphinucleolus. 7, Granular Band I. 8, Granular Band II, mitochondria and proteid yolk. 9, Dense proteid yolk. 10, Centrifugal vacuole.

reagent. When the eggs were previously extracted with 4% trichloracetic acid at 90° for 15 minutes, the nucleolus remained unstained following the Feulgen test.

Kobayashi (1953, 1954) observed similar amphinucleoli in the eggs of the oyster (*Ostrea laperousi*) which were Feulgen-positive. He also found that the karyosome stained with methyl green and the plasmosome with pyronin, indicating the presence of RNA.

Another type of amphinucleolus was reported by Sawada and Murakami (1959) in *Mactra veneriformis*. In this form, nucleolus 1 stained deeply with pyronin while nucleolus 2 gave a weak reaction with pyronin.

Attempts to demonstrate RNA in the non-staining intranucleolar buds of the mature oocytes in *Pectinaria* were unsuccessful, while in very young oocytes both epinucleolar and intranucleolar buds fluoresced bright red with acridine orange and

stained metachromatically with thionin. Acridine orange stained RNA nucleoli in tumor cells bright red (Tweedell, 1960a).

Changes in the amphinucleoli during the growth phase are well known in molluscan eggs; Raven (1958) and Wilson (1925) report on numerous cases of variation in the amphinucleoli of annelids, molluscs and arthropods. This appears to be the case in the nucleoli of the developing oocytes of *Pectinaria*.

Summary

1. Living eggs of *Pectinaria gouldii* were stained with vital dyes and vital fluorochromes before and after germinal vesicle breakdown. Observations were made with the bright field, dark field and fluorescence microscopes. Changes in the germinal vesicle, nucleolus and chromosomes of the living eggs were followed.

2. Other eggs were vitally dyed and centrifuged in order to stratify the cell particulates. Cell granules were identified, based upon their specific staining and their stratified position in the centrifuged egg. These included lipid droplets, lipid granules, mitochondria, proteid yolk, basophilic and cortical granules. Limited cytochemical tests were made to verify their identity.

3. Two kinds of metachromatic granules were seen. With toluidine blue, the granules occur at the centripetal pole just beneath the lipid cap. When Nile blue sulphate was applied, a different metachromatic band stratified just centripetal to the heavier yolk granules. Astral granules originate from the latter metachromatic band and became associated with the first maturation spindle.

4. The fluorescent cell components included cell and nuclear membranes, nucleoli, chromosomes, lipid granules, two types of yolk granules and mitochondria.

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